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Effect of Increased Activity on Metabolic Markers in Captive Black Rhinos: A Pilot Study

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June 3, 2009

ACKNOWLEDGEMENTS

I would like to extend special thanks to Alisa Sandor, head black rhino keeper at the Cleveland Metroparks Zoo. Further thanks is extended to relief keepers Kevin Scotti, Heather Strawn, Joe Conrad, and Meghan, and student volunteers Jeff, Deb, Gina, Molly, Marjie, and Poorna who dedicated the extra hours to exercising rhinos.

ABSTRACT

Ex situ (captive) black rhinoceros populations exhibit higher rates of disease than free-ranging counterparts. Preliminary data from an ongoing study supports the association between excessive adipose tissue, iron overload, insulin resistance and increased inflammatory markers in captive animals. This study hypothesizes that an increase in activity level by 30 minutes daily at a moderate activity level for 8 weeks will result in improvement in insulin sensitivity (measured by a decrease in the serum insulin to glucose ratio) and decreased inflammation (measured by TNF-alpha and serum amyloid A). Serum markers of body condition (leptin), inflammation (TNF-alpha, serum amyloid A), insulin sensitivity (insulin, glucose), phosphate and iron stores (ferritin) will further be measured.

An adult male and female at the Cleveland Metroparks Zoo are the subjects for this pilot study. ELISA assays have been validated for measuring serum insulin, ferritin, TNF-α, and serum amyloid A in black rhino. These assays were used to assess changes in markers of metabolic status from weekly serum samples collected during 2 weeks of baseline and 10 weeks of increased activity. Methods to increase activity include delaying feeding times by 30 minutes when the subject is already in motion, a call and reward system, playing fetch, and jogging in the yard. The GT1 Actigraph, an accelerometer commonly used in field research, has been used to establish baseline activity levels and measured activity levels during the ten week interval of increased exercise. Our studies demonstrated a significant ($P<0.001$) increase in activity level above baseline in both the male and female black rhino. Serum metabolic markers

indicate decreases in inflammation and iron stores, with no significant changes in insulin sensitivity.

Key Words: Black rhinoceros, metabolic disorders, hypophosphatemia, accelerometry, ELISA assays

BACKGROUND

Since 1960, numbers of wild black rhinoceroses (*Diceros bicornis*) have declined from 100,000 to approximately 4,000 (IRF, 2008). While wild conservation programs are critical for the survival of the species, proper management of the 247 captive rhinos worldwide is also a key element to species conservation (IRF, 2008). Unfortunately, *ex situ* populations exhibit disease syndromes not identified in free-ranging black rhinoceros populations (Dennis et al., 2007). Of approximately 300 captive black rhinos surveyed that died between 1930 and 2001, 73% did not reproduce. 77% of these animals never reached sexual maturity (Dennis, 2004). Therefore, captive breeding programs are not self-sustaining.

Problem Identification and Justification

Metabolic disorders observed in captive black rhinos include hemolytic anemia, idiopathic hemorrhagic vasculopathy, liver disease, hepatopathy, rhabdomyolysis, leukoencephalomalacia (neurological symptoms), and necrolytic dermatopathy (skin lesions) (Dennis et al., 2007). Though these syndromes are the leading causes of death in captive black rhinos, their etiologies remain unknown (Dennis et al, 2007).

Hemosiderosis in several major organs is commonly reported on necropsy. Mammals lack an effective method for excreting iron, and speculated dietary contributions to iron overload include a captive diet that has both higher levels of iron and fewer iron-binding substances (Dennis et al., 2007). Further, genetic evidence suggests a possible mutation that would allow for more efficient iron absorption in black rhinos compared to other rhinoceros species (Beutler et al., 2001). Previous studies have attributed rhinoceros metabolic disorder pathology to iron overload and impaired

antioxidant capacity, leading to free radical production and oxidative stress (Paglia et al., 1996, Paglia et al., 2000). While hemosiderosis is associated with disease in humans, none of the diseases are similar to those observed in black rhinos (Dennis et al., 2007).

The investigators instead hypothesize that iron overload is a consequence of other underlying causes of disease. The hypothesis is supported by studies in humans and mice where excessive adipose tissue increases inflammation (Hotamisligil, 1993, Rajala and Scherer, 2003). Both of these events contribute to iron overload, insulin resistance, and hypophosphatemia (Dennis et al., 2007, Gaasbeck and Meinders, 2005) (Fig. 1).

Fig. 1. Cascade of metabolic events leading to disease.

In obese humans, the adipose tissue population shifts to increase macrophage activation and recruitment. This shift causes a chronic inflammatory state, increasing circulating levels of acute phase proteins and inflammatory cytokines. Inflammatory cytokines have a crucial role in the development of insulin resistance by (1) inhibiting insulin signaling and glucose uptake, contributing to hyperglycemia and (2) interfering with insulin's inhibition of glucose production in the liver by contributing to iron overload through the stimulation of cellular iron uptake with the up-regulation of ferritin. Insulin stimulates cellular phosphate absorption, and hyperphosphatemia exerts a negative feedback on glucose transport, contributing to more hyperglycemia. With hypophosphatemia comes a depletion of adenosine triphosphate (ATP) and 2,3-diphophoglycerate (2,3-DPG), the molecule that modulates oxygen release by hemoglobin.

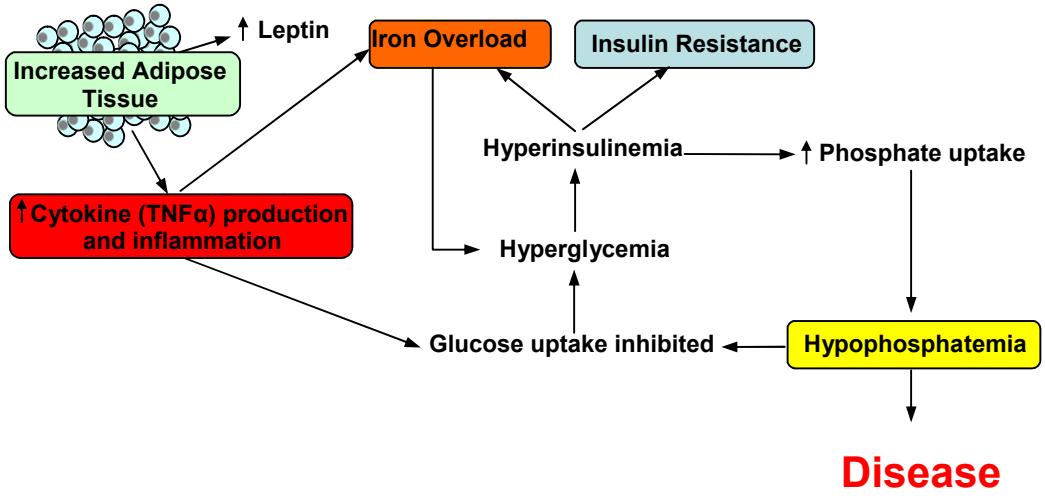
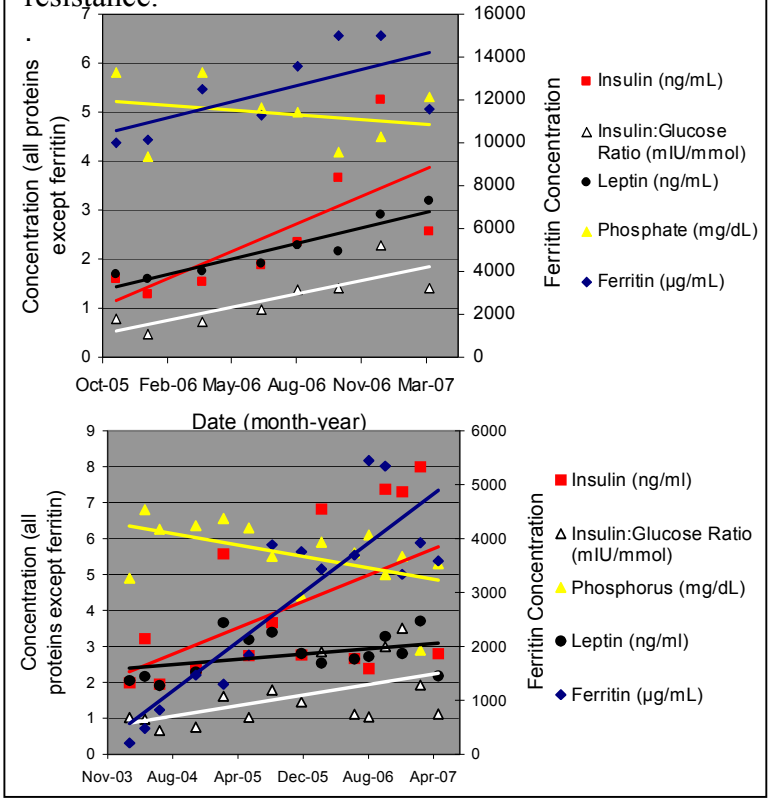
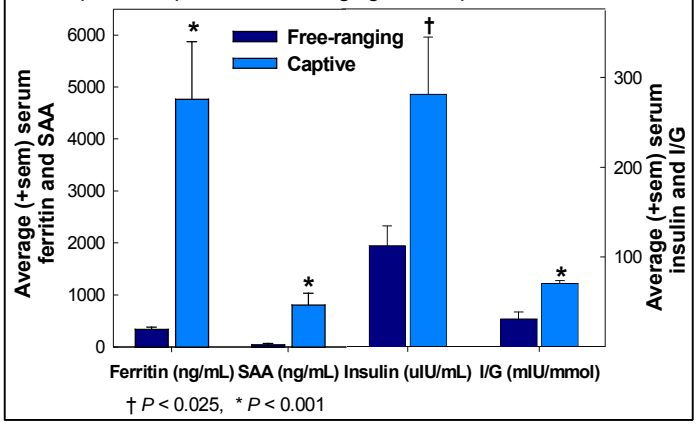


Fig. 2. Ferritin, leptin, insulin, and insulin:glucose ratio increase over time while phosphate decreases in one male (panel A) and one female (panel B) captive black rhino, indicating increased body condition and insulin resistance.



Hypophosphatemia is further correlated with hemolytic anemia, rhabdomyolysis, encephalopathy and acute liver failure in humans (Nanji and Anderson, 1985). Previous studies note hypophosphatemia in cases of black rhinoceros illness (Murray et al, 1999, Paglia et al., 1996, Paglia, 1993). Preliminary data from an ongoing study supports the association between excessive adipose tissue, iron overload, and increased inflammatory markers with time in captivity (Fig. 2). The ongoing study is further looking into the specifics of serum hormone levels in 10 captive and 30 free-ranging black rhinos, and has determined significant differences in iron stores, inflammation, and insulin sensitivity in captive animals (Fig. 3).

Fig. 3. Measures of iron stores (ferritin), inflammation (SAA) and insulin resistance [insulin, insulin:glucose ratio (I/G)] are increased in captive compared to free-ranging counterparts.



INTRODUCTION TO PILOT STUDY

As a corollary to the ongoing study, this pilot study postulates that captive black rhinos are susceptible to inadequate physical activity, excessive caloric intake, and increased fat stores. The study specifically investigates the effect of management changes to increase activity level (by 30 min daily at a moderate level of activity) on serum levels of inflammation [tumor necrosis factor alpha (TNF-alpha) and serum amyloid A (SAA)], insulin sensitivity (insulin-to-glucose ratio), phosphate and iron stores (ferritin). The link between obesity and insulin resistance in humans has led to the use of exercise to decrease insulin resistance and adiposity (Hawley and Lessard, 2008). In designing exercise programs, it has been demonstrated that duration of training is an important factor in increasing insulin sensitivity (Houmard et al., 2004). Even when individuals maintain current adipose levels, exercise is still shown to increase insulin sensitivity (Duncan et al., 2003). The information in this study is intended to encourage other Association of Zoos and Aquariums (AZA) accredited zoos to enroll their black rhinos in a larger, more comprehensive study from which statistically significant correlations can be drawn.

Hypotheses/Objectives

An increase in activity level by 30 minutes daily at a moderate activity level for 10 weeks will result in improvement in insulin sensitivity (measured by a decrease in the serum insulin to glucose ratio) and decreased inflammation (measured by TNF-alpha and SAA).

MATERIALS AND METHODS

The activity of an adult male and female at the Cleveland Metroparks Zoo was increased by 30 minutes a day for 10 weeks (72 days). Measurements were taken in order to assess (1) changes in physical activity levels and (2) concomitant changes in metabolic health. Further, the feasibility of increased activity protocols was evaluated.

Methods to Increase Physical Activity

The male and female subjects were housed separately from each other and could not be exercised simultaneously. Each subject required different methods to increase their activity levels. Captive black rhinos are managed in a protected-contact setting. This implies that while it is possible to directly touch, feed, and interact with the animals, all contact occurs from behind a protective barrier. Both zookeepers and student volunteers were responsible for implementing increased activity protocols. While all of the zookeepers had animal training experience, student volunteers did not. Therefore, all methods used were (1) in compliance with protected contact protocols and (2) easily taught to persons without animal training experience.

Of the two subjects, the male subject required less management time to increase his activity. In the morning, the male subject usually remained in motion until given a meal. By postponing his first meal by 30 minutes, the male effectively increased his activity above baseline levels. Of the 72 days in the study, 52 days (72.2%) had the entire 30 minute exercise requirement filled by this technique. All other exercise was achieved through call and reward as outlined below in the female subject.

The female subject required several different methods to maintain her interest in activity. The technique most commonly used was “call and reward.” In the indoor

exhibit, the female subject was separated from other rhinos and given 2 to 3 stalls for the duration of a session. The female was rewarded upon coming when called. Rewards includedbrushing, fibrous branches (a favored food item) and very small (<1/2 to 1 in^3) pieces of produce, herbivore pellets, and alfalfa cubes. These were part of her regular diet, and were not intended as additional calories. During a call and reward session, it was possible to have the female subject retrieve a stick thrown to her. While this was an effective supplement to a call and reward session, it could not maintain the female's interest for a full 30 minutes.

It was further possible to achieve a more intense level of exercise by having a keeper or student volunteer jog along the perimeter of the outdoor exhibit. While the female subject would occasionally run with the keeper or student volunteer, she would become easily distracted in the large yard. Sessions were further shortened if the female subject's mother, the dominant animal, was present in the yard. Therefore, outdoor exercise was most effective when the female subject and her younger sister were alone on exhibit, thus encouraging more intense running. Of the 72 days in the study, only 7 days (9.7%) had one or more sessions completed in the outdoor exhibit. All other exercise was achieved through call and reward.

It is worthy to note that the length of an exercise session was limited by the rhino's participation. If the rhino stopped moving for approximately 30 seconds, a stopwatch timer was stopped and only restarted when the rhino continued its activity. The session would end if the rhino did not regain interest in the exercise activity within ~5 minutes. While it was sometimes possible accomplish the full 30 minutes of exercise in one session, exercise was commonly split into 2 or 3 shorter sessions (Table 3)

Table 3. Number and percentage of days requiring either 1, 2 or 3 sessions to achieve 30 min of exercise. 0 sessions indicates that no exercise occurred.

Male

Number of sessions achieved in 1 day	Number of days	% of days
0	3	4.2
1	60	83
2	7	9.7
3	2	2.7
Sum	72	99.6

Female

Number of sessions achieved in 1 day	Number of days	% of days
0	4	5.6
1	31	43
2	34	47
3	3	4.2
Sum	72	99.8

Measuring Physical Activity

Activity was recorded using two methods. Accelorometry was used to quantitatively determine intensity and amount of exercise, while an activity log was used to record information about exercise sessions. Because the focus of the study was on increased activity levels and not behavior per se, no behavioral observations were collected.

Accelerometry is a valid and time efficient tool that can be used to measure both distance travelled and energy expenditure (Sellers and Crompton, 2004, Welk et al., 2004). Actigraph accelerometers measure activity "counts" in a 1-minute interval (Treuth et al., 2004). The Actigraph company (Pensacola, FL) defines counts as <2,000/min as 'light' activity, 2,000 to 6,000 as 'moderate', 6,000 to 9,000 as 'hard' and >9,000 as 'very hard.' When calibrated against oxygen consumption (VO_2) measures of physical activity in adolescent

girls, thresholds were defined as watching TV or playing a computer game for sedentary activity, sweeping the floor for light activity, walking slowly for moderate activity, and jogging/running for hard/very hard activity (Treuth et al., 2004). Because moderate-to-vigorous levels result in positive health changes, including lower circulating insulin levels, (Treuth et al., 2004) this study attempted to increase the number of minutes of 'moderate' or higher activity (>2,000 counts min^{-1}) achieved each day by 30 minutes.

The GT1 Actigraph (Manufacturing Technology, Inc., Fort Walton Beach, FL), was used to establish baseline activity levels and activity patterns during a two week (n=14) control period and measure increased activity during the 10-week (n=72) experimental period. Thus, each animal served as its own control. While the battery life of each Actigraph device was 2 weeks, devices were changed on a weekly basis in order to monitor data collection and ensure the integrity of the device. The device was sealed in two waterproof bags and attached comfortably to the front leg using a lightweight, canvas fire hose anklet (Fig. 4). Anklets could be easily fastened and removed from both subjects without physical or chemical restraint.

Fig. 4. Actigraph enclosed in light-weight canvas firehouse.

. The quality of each exercise bout was ranked on a subjective 5-point scale (Table 3) for the purposes of subjectively recording the intensity observed in each exercise session. The time of day, a number representing intensity, and length of each session were recorded in an activity log.

Table 3. Subjective 1-5 scale describing quality of exercise sessions

1	2	3	4	5
Disinterested/ Frequent stopping Sluggish pace	Sporadic interest Some stopping Plodding pace	Steady interest Little stopping Steady pace	Steady interest Little stopping Brisk walking	Steady interest Little stopping Fast paced/ Jogging

From this information, it was possible to quickly assess and compare the effectiveness of increased activity protocols throughout the 10-week interval. Further, information from the activity log could be used to describe basic animal husbandry methods relating to the trial period, i.e., number of sessions required per day or the exhibit exercise occurred in.

Measuring Changes in Metabolic Markers

On the first day of each one-week interval, a blood sample (12 ml) was obtained from each (unrestrained, non-sedated) rhino by venipuncture from a leg vein. Serum was harvested and stored at -20^{o}C after allowing blood to clot and centrifuging (3,000 X g, 10 min). Samples were collected during both baseline and trial period weeks. Serum concentrations of markers were measured in order to assess weekly changes in inflammation, insulin resistance, and iron stores.

Inflammation was measured by using the markers TNFα, an inflammatory cytokine, and the acute phase protein SAA. TNFα contributes directly to insulin resistance and promotes iron storage (Ruan and Lodish, 2003, Yanoff et al., 2007). SAA contributes to insulin resistance and correlates to body mass index in humans (Xu et al., 2003, Yang et al., 2006). While frequently sampled intravenous glucose tolerance test (FSIGT) is the preferred, most accurate measure of insulin sensitivity in humans, (Uusitupa et al., 2003, Duncan et al., 2003), it is impractical for use in black rhinos,

requiring vein cannulation and continuous sampling over 3 hours. Further, an animal with a hind-gut fermentation digestive system would need to be fasted for days before accurate measurements could be made. Therefore, corrections for blood glucose were instead measured as insulin to glucose ratios. Ferritin, the protein-bound storage form of iron is significantly correlated with iron stores in horses (Smith et al., 1984).

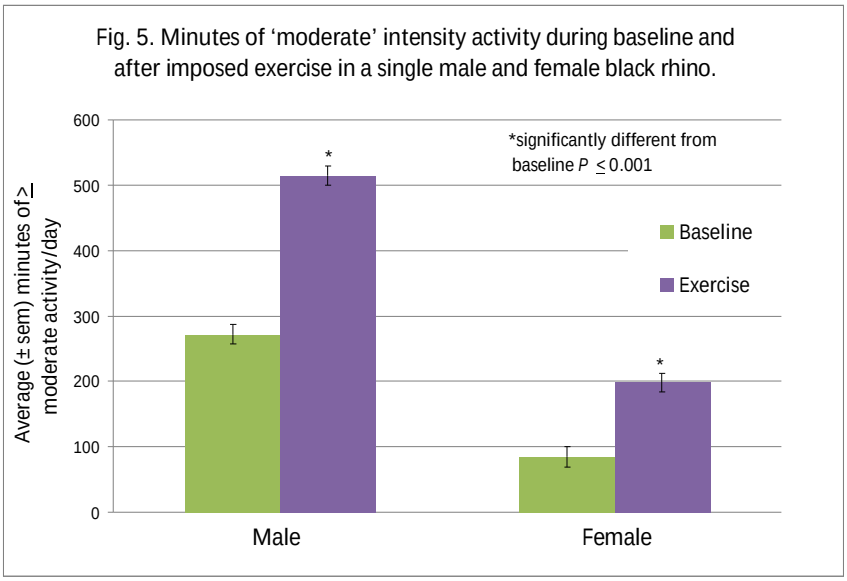
Commercial enzyme immunoassays were validated for black rhino serum and were used to measure insulin, TNF-alpha, and serum amyloid A. Dr. Mandi Vick developed and validated a ferritin assay based upon an assay developed for horses that exhibited linearity and parallelism with the standard curve (Smith et al., 1984). Standard veterinary blood chemistry analyzers were used to determine serum glucose concentrations. General linear model analysis was used to evaluate changes over time in each marker over the duration of the trial period.

RESULTS

Accelerometry

In establishing baseline activity levels, patterns of greatest inactivity were noted for the two animals. The female was least active between the hours of 7:00-8:00 and 12:00-15:00. The male was consistently inactive throughout the day. Based on the baseline activity levels, these hours of least activity were selected as the times during which to conduct the exercise sessions.

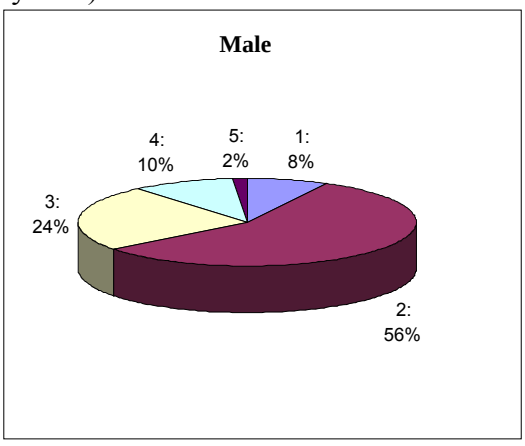
By conducting exercise sessions during these times, statistically significant, quantifiable increases in the daily number of minutes of moderate activity ($P<0.001$) were achieved (Fig. 5)

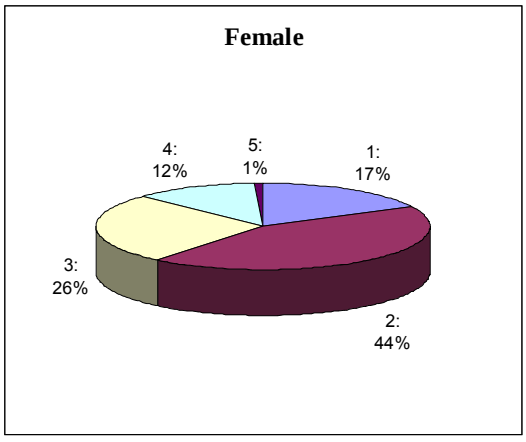


Activity Log Calendar

Throughout the 72 day duration of the study, 3,953 minutes of exercise were facilitated by either keepers or student volunteers. In general, most activity minutes (88%) fell at or below the threshold of 3: "steady interest, little stopping, steady pace."

Fig. 6. Distribution of exercise minute intensity (As described by subjective scoring system)





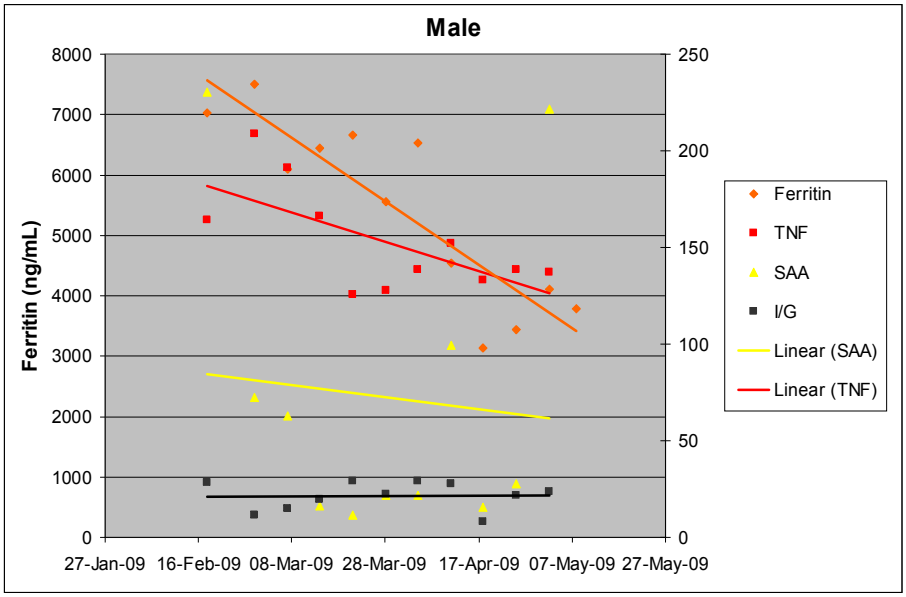
Changes in Metabolic Markers

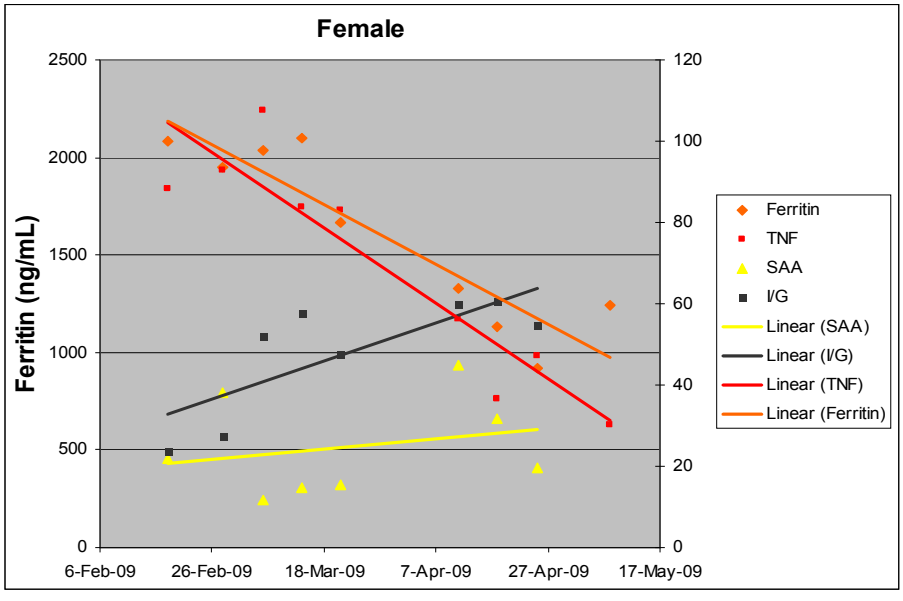
Two baseline serum samples were collected from each subject. All ten weekly increased activity samples were collected from the male, but only 7 weekly increased activity samples could be collected from the female. Using general linear model analysis, several trends were observed (Fig 7). First, ferritin demonstrated a significant ($P<0.05$) decrease over time in both subjects. Second, the inflammatory marker TNF alpha demonstrated a decreasing trend over time, yet not of any statistical significance. SAA and insulin to glucose ratio decreased over time in the male subject but showed an increasing trend in the female subject. None of these trends were of statistical significance. No trend was indicated in either subject for serum phosphorus levels over time (data not shown).

The lack of statistically significant trends over time for inflammatory markers and insulin to glucose ratio could be a result of several factors. First, both a greater intensity (high or very high) and/or number of minutes of exercise per day may need to be achieved before statistically significant decreases in inflammatory markers and insulin to glucose ratios can be achieved. Second, the activity period of 10 weeks may not have been a sufficient length of time to observe the hypothesized trends. Third, while attempts were made to keep the size of rewards to a minimum in call and reward sessions, there was no quantitative way to control for feed intake. It is possible that the subjects ate more calories than they were exercising, leading to a net caloric increase and subsequent increases in the amount of adipose tissue producing inflammatory cytokines. Fourth, there may not be any merit to the hypothesis that the described cascade of events leading to disease in black rhinos could be mitigated by exercise.

The significant decrease in ferritin over time indicates that there was some metabolic improvement with increased activity, making the first four factors more likely explanations for the lack of statistically significant trends observed for measures of inflammation and insulin sensitivity. The lack of a statistically significant trend was partially expected for phosphorus because moderate cases of hypophosphatemia are generally asymptomatic (Barak et al, 1998).

Fig. 7. Observed trends in metabolic markers over time





CHALLENGES AND AREAS FOR FUTURE STUDY

The methods and techniques described in this pilot study were meant as a starting point from which future increased activity projects could develop. Several major areas for the improvement of future studies are outlined below. After making adjustments, it is ultimately hoped that at least 10 black rhinos from AZA accredited institutions will participate in a larger, more comprehensive increased activity study.

Exercise techniques

While the activity log included the start time of exercise sessions, it excluded an end time to the session. A 30 minute session frequently took 40 minutes or longer to achieve due to poor rhino participation. Therefore, there was no way to accurately correlate a specific exercise session's subjective score with Actigraph data. If a significant relationship is shown to exist between exercise and metabolic health in black rhinos, it would be time and cost effective to have already quantitatively evaluated the efficacy of different exercise methods.

Each of the two major exercise techniques presented their own challenges. By postponing the male subject's first meal, the concern is raised as to whether or not this encouraged pre-meal stereotypic pacing. Though delaying a meal was apparently the more effective and time efficient method to increase activity, behavioral studies would likely need to be completed to assess whether this method induced adverse behaviors (or stereotypic behaviors) or had long-term adverse effects on the animal.

The biggest challenge of the call and reward method was the time commitment from the keepers. It frequently took an hour of keeper time to achieve 30 minutes of activity. Student volunteers were able to decrease the time required from the keepers, but

were only available a maximum of 5 days a week. Further, student volunteers did not have any animal training experience and were often faced with the challenge of exercising an unresponsive rhino.

Lastly, it was almost impossible to guarantee that net caloric intake did not increase with the amount of rewards given. Even if total rewards given were weighed, very little information could be gained because caloric values vary for different rewards. Weighing rewards would have also required more management time from the keepers.

Future Investigation

Therefore, the second phase of this study will attempt to increase activity by varying the time and location that meals are fed. It is hoped that this method will increase activity to levels achieved in this pilot study (or higher) without possibly encouraging stereotypic behaviors, requiring as much keeper time or potentially increasing net caloric intake.

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